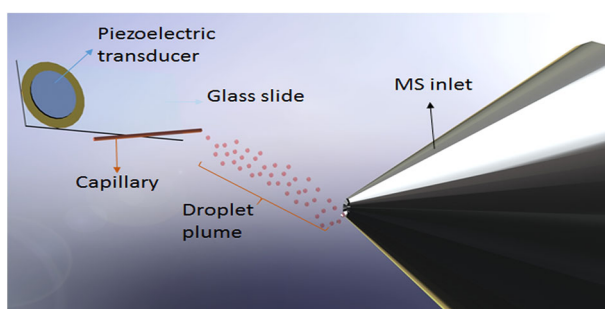


Capillary Vibrating Sharp-Edge Spray Ionization (cVSSI) for Voltage-Free Liquid Chromatography-Mass Spectrometry

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Abstract. Here, we report a continuous flow-based ionization method, capillary vibrating sharp-edge spray ionization (cVSSI), that nebulizes liquid sample directly at the outlet of a capillary without using high-speed nebulization gas or a high electrical field. cVSSI is built upon the recently reported VSSI principle which nebulizes bulk liquid using vibrating sharp-edges. By attaching a short piece of fused silica capillary on top of the vibrating glass slide in VSSI, liquid is nebulized at the outlet

of the capillary as the result of the vibration. Utilizing standard 360- μm OD/100- μm ID capillary, cVSSI works with a wide range of flow rates from 1 $\mu\text{L}/\text{min}$ to 1 mL/min . The power consumption is as low as 130 mW. ESI-like MS spectra are obtained for small molecules, peptides, and proteins. Five orders of magnitude linear response for acetaminophen solution is achieved with a limit of detection (LOD) of 3 nM. cVSSI is also demonstrated to be compatible with LC-MS analysis. Two LC-MS applications are demonstrated with cVSSI: (1) separation and detection of a mixture of small molecules and (2) bottom-up proteomics using a protein digest. A mixture of nine common metabolites was appropriately separated and detected using LC-cVSSI-MS. In the bottom-up experiment, 78 peptides were detected using LC-cVSSI-MS/MS with a protein coverage of 100% for cytochrome *c*, which is comparable with the coverage obtained using LC-ESI-MS. cVSSI offers a means of interfacing LC or other continuous flow-based applications to mass spectrometers with the salient features of voltage-free, flexibility, small footprint, and low power consumption.

Keywords: Ionization, Mass spectrometry, LC-MS, Voltage-free nebulization

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Introduction

Mass spectrometry (MS) is a robust tool to detect and quantify molecules with a high degree of accuracy, resolution, and information available for appropriate data

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processing [1, 2]. MS technologies have been widely applied to diverse fields [3], including chemical analysis, forensics [4–6], plant sciences [7–9], materials [10–13], and basic life sciences [14–20]. In these areas of application, there is often a need for chemicals or biomolecules to be separated from a mixture using liquid chromatography (LC) methods prior to MS analysis. Current standard LC-MS systems use electrospray ionization (ESI) [21] to produce gas-phase ions of sample analyte as ESI is compatible with the fluidic system of LC [22]. While ESI has been widely used in many LC-MS applications, for chemicals that are sensitive to electrochemical reactions or biomolecules that need to be

investigated in the absence of a high voltage, a voltage-free ionization method would be more desired [23–25]. To date, several voltage-free ionization methods have been coupled to LC systems including thermospray, sonic spray ionization (SSI), and solvent-assisted inlet ionization (SAII) [26–29]. In addition, ultrasonic agitation has also been demonstrated as an effective voltage-free ionization strategy with the advantages of small system footprint, high flexibility, and ease of use [30–33]. However, existing mechanical ultrasonic ionization methods are difficult to integrate with LC systems as these methods require liquid samples to be placed on a substrate surface, which is not directly compatible with a LC fluidic system. Although surface acoustic wave nebulization (SAWN) has been coupled to a LC by placing the outlet of the LC capillary tubing on top of the SAW substrate for peptide analysis, this discrete coupling strategy is prone to dead volume, carryover, and injection variation effects [33]. As a result, the optimal flow rate range for this setup is limited (1–5 $\mu\text{L}/\text{min}$). Here, we report a new mechanical vibration-based ionization method, capillary vibrating sharp-edge spray ionization (cVSSI), that can directly nebulize and ionize LC eluent at the end of a capillary thereby eliminating the issues caused by previous discrete connection methods.

The cVSSI is built upon the recently reported vibrating sharp-edge spray ionization (VSSI) principle [34]. In VSSI, liquid samples are nebulized at sharp-edges of a vibrating glass slide, which produces ESI-like mass spectra. VSSI offers a convenient way of ionizing target molecules for direct MS analysis due to its simplicity and flexibility. One disadvantage with the glass slide platform in VSSI is the difficulty for continuous flow injection analysis. Thus, it is not suitable for direct coupling with LC or other continuous flow-based MS analyses. Here, we report a modified VSSI method, cVSSI, that can nebulize liquid samples directly at the outlet of a fused silica capillary based on the VSSI principle, which allows for continuous flow-based injection.

cVSSI consists of a capillary attached to a glass slide, which can be vibrated by a piezoelectric transducer (Figure 1). Once liquid reaches the outlet of the vibrating capillary, it is subsequently nebulized and directed to the inlet of the mass spectrometer. The capillary setup improves the signal stability, reproducibility, and quantification capability over the previous VSSI method. Five orders of magnitude linear response for acetaminophen solution are achieved using cVSSI with a limit of detection (LOD) of 3 nM. More importantly, cVSSI enables the direct coupling of LC separation with MS analysis. We demonstrate two typical applications of LC-MS analysis with cVSSI including the analysis of a metabolite mixture and a bottom-up proteomics analysis of a protein digestion. The cVSSI method offers a new means of generating a microdroplet plume directly from the capillary outlet without the need of a nebulizing gas and electrical field, which greatly simplifies the setup and reduces the footprint of the ionization unit for continuous flow-based MS analysis.

Experimental Section

Materials and Reagents

High-performance liquid chromatography (HPLC)-grade acetonitrile, water, ammonium acetate, and formic acid were purchased from Fischer Scientific (NJ, USA). Sucrose, caffeine, acetaminophen, dopamine, and acetic acid were purchased from Sigma-Aldrich (MO, USA). Benzoic acid was purchased from Fischer Scientific. Guanine and guanosine were purchased from Acros Organics (NJ, USA). Angiotensin I was purchased from Alfa Aesar (MA, USA). Ubiquitin was purchased from Boston Biochem (MA, USA). Cytochrome *c* (equine) and pepsin (porcine, ≥ 2500 units/mg protein) were purchased from Sigma-Aldrich (MO, USA) and used without further purification.

Instrument

A high-performance Q-Exactive (ThermoScientific) mass spectrometer featuring quadrupole precursor selection with high-resolution, accurate-mass (HR/AM) Orbitrap detection was used for all MS analyses. The Accela ultra high-performance liquid chromatography system (ThermoScientific) was used for LC separation experiments which also features a photodiode array detector for absorbance measurements across multiple wavelengths. Measurements were carried out under ambient conditions and employed a capillary inlet temperature of 250 °C. A Model Fusion 200 syringe pump from Chemyx Inc. was used to pump analytes at set flow rates.

cVSSI Fabrication and Operation

cVSSI devices were fabricated by attaching a short piece (~ 5.5 cm) of fused silica capillary (Polymicro Technologies) on the distal end of a VSSI device. The capillary is immobilized on the VSSI glass slide by glass glue. A detailed fabrication of the VSSI device is described in our previous work. Briefly, a piezoelectric diaphragm (Murata) is bonded to one end of a no. 1 microscope glass slide (VWR) using epoxy-based super glue (Devcon).

The cVSSI device is activated using a function generator (Tektronix) and a power amplifier (Mini-Circuits) at frequencies of 93–97 kHz. For direct MS analysis, cVSSI is connected with 30-gauge PTFE tubing for sample introduction. The device was nominally placed in line with the inlet of the mass spectrometer at a distance of ~ 5 mm. For LC-MS experiments, cVSSI is connected to the LC fluidic system via a MicroTight Union connector (Upchurch).

LC-MS and LC-MS/MS Analysis

A reversed-phase C18 analytical column (ThermoFisher Scientific, catalog number 17626-052130), 2.1-mm diameter, 50-mm length, 2.6- μm particle size, and pore size 80 Å, was used for LC separation. 0.1% formic acid in water (buffer A) and HPLC grade methanol (buffer B) were used as the aqueous and

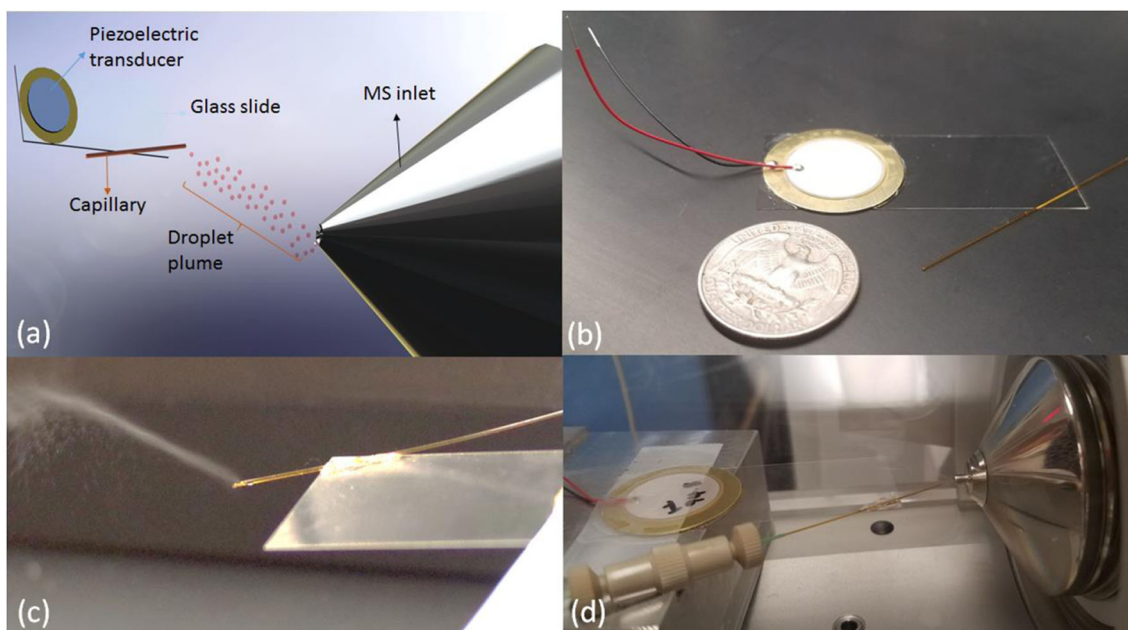


Figure 1. (a) Schematic of cVSSI-MS. (b) Real image of cVSSI device. (c) Plume generation at the outlet of the capillary in cVSSI. (d) cVSSI setup at the inlet of the mass spectrometer with a capillary connector

organic phase buffer solutions. The separation flow rate was set at 500 $\mu\text{L}/\text{min}$. A gradient program for the metabolites' separation is listed as follows: start 5.0% B; 1.5 min 5.0% B; 8.5 min 30% B; 11.5 min 100% B; 14.5 min 100% B; 17.5 min 5% B. For each injection, 20 μL of sample was used. The eluant from the HPLC column was split to reduce the load on the cVSSI device using a 10:1 split. Therefore, the eluant passed through the cVSSI device at a rate of 50 $\mu\text{L}/\text{min}$ (down from 500 $\mu\text{L}/\text{min}$ through the column). The flow was split at the end of the HPLC column using a T-connector in which one-tenth of the length of the fused silica capillary from the T-connector to the cVSSI was connected to waste. The remaining solvent flowed through the cVSSI device. The cVSSI flow rate was determined by measuring the volume of the collected solvent obtained when the device was not operating.

The same reversed-phase column, solvent buffers, and flow rate were employed for the protein digest separation. The peptic digestion of cytochrome *c* was achieved by dissolving 10 mg of cytochrome *c* in 1.0 mL of pure water with subsequent addition of 0.5 mg of pepsin. The solution was then acidified to a pH of ~ 3.5 by dropwise addition of acetic acid. A gradient program for the peptide separation is listed as follows: start 5.0% B; 1.0 min 5.0% B; 18 min 50% B; 21 min 100% B; 24 min 100% B; 27 min 5% B; 30 min 5% B. For each injection, 20 μL of sample was used. The flow was split 10:1 for the cVSSI into the mass spectrometer.

The mass spectrometer settings for the two different analyses were as follows. For the full/precursor ion mass spectrum collection in the LC-MS and LC-MS/MS analyses, a resolving power of 7×10^4 was used. For the LC-MS/MS analysis, the data-dependent approach used a resolving power of 1.75×10^4 for the fragment ion spectra and a m/z isolation window of 5. A value of 5 was used for

the maximum number of MS/MS spectra to be acquired between precursor ion MS spectra collection.

Results and Discussion

Nebulization at the Capillary Tip

In our previous report, liquid is nebulized at the sharp-edge of a vibrating glass slide [34]. We speculated that the high vibration amplitude and the sharp-edge-induced acoustic streaming are the main contributor to this phenomenon. Therefore, we hypothesize that if we can generate a similar vibration at the outlet of a capillary, nebulization with a capillary can also be achieved without the need of a high-voltage electrical field, a high-speed nebulizing gas, or a vacuum system. To generate effective vibration of a glass capillary, we sought to modify our previous VSSI setup by attaching a short piece of fused silica capillary (5.5 cm in length, 100- μm ID, and 360- μm OD) on the distal end of a VSSI glass slide as shown in Figure 2. By applying ~ 93 kHz signal to the transducer, we observed plume generation when water was pumped through the capillary at a flow rate of 20 $\mu\text{L}/\text{min}$ with a power input ~ 260 mW. To generate efficient vibration at the tip of capillary, the relevant position between the capillary and the glass slide requires optimization. With the VSSI setup, the distal end of the glass slide allows for the highest vibration amplitude, so we first attached the capillary onto the distal end of the glass slide using epoxy glue (Figure 2a). To ensure optimal vibration at the outlet of the capillary, the total mass extended beyond the glass slide was minimized. When the capillary extends ~ 4 cm beyond the glass slide (Figure 2b), nebulization efficiency decreases due to a damping effect. The outlet of the capillary also should not be placed too close to the end of the glass slide,

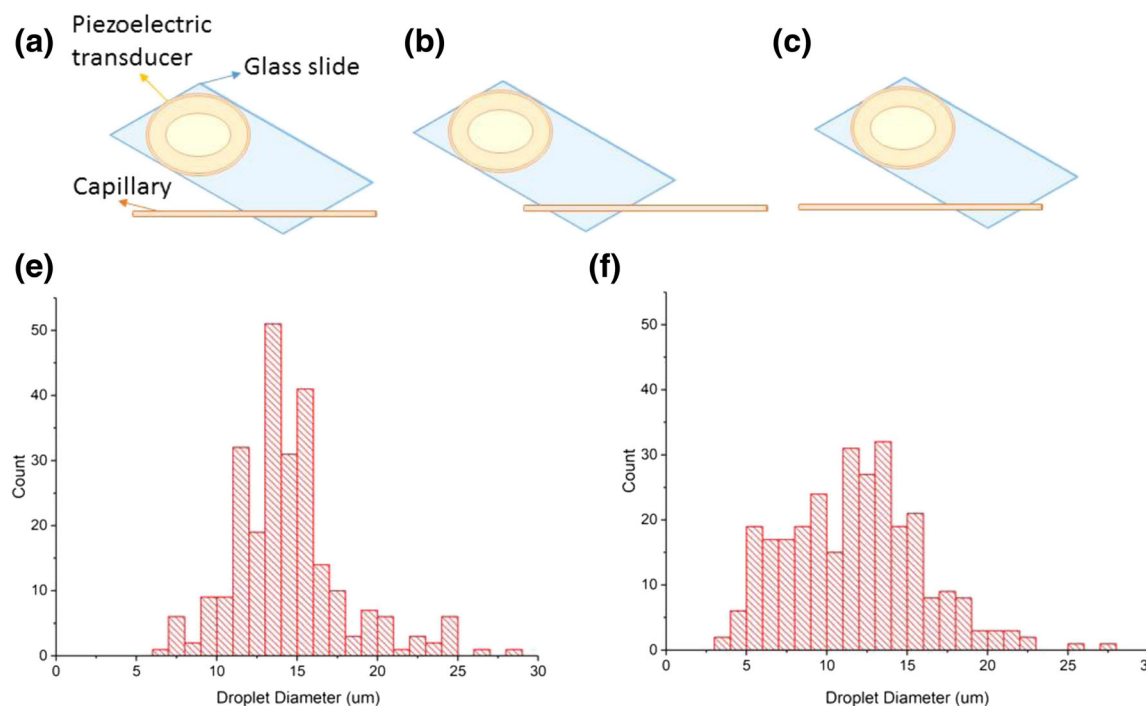


Figure 2. (a) Optimal position for capillary attachment on the glass slide. (b) and (c) Non-optimal positions for capillary attachment that damp plume generation or cause unstable plume generation. (e) and (f) Droplet size distribution for VSSI and cVSSI, respectively

which causes instable spray as liquid can flow back onto the glass slide (Figure 2c). We determined the optimal extension for the outlet end of the capillary was ~ 1 cm, which can maintain a stable spray while requiring small power input. We also found that the length of the inlet end of the capillary has minimal impact on the nebulization. This feature allows us to maintain a relatively long inlet end (~ 4 cm) for convenient tubing connection. The inlet end of the capillary can be connected to plastic tubing directly for low-pressure injection or connected to a capillary connector for high-pressure applications, e.g., LC-MS, without affecting the nebulization at the capillary outlet. Compared with direct VSSI, we noticed a frequency shift from 97 kHz to 93 kHz in cVSSI experiments due to the added mass in cVSSI. We also compared the droplet sizes generated by cVSSI and VSSI using water, which shows a similar size distribution (Figure 2e, f) for both setups. An Olympus IX73 inverted microscope was used for droplet size characterization under 10x magnification. In cVSSI, the average size of droplets is 12 ± 5 μm , which is determined by capturing the droplets from the plume with mineral oil and subsequently examining aqueous droplets under a microscope.

We also studied the size of the capillary and its impact on plume generation. In addition to the 100- μm ID capillary, 50-, 75-, and 250- μm ID capillaries were also tested. All of these capillaries worked for cVSSI under the proper power input. Generally, as the ID of the capillary decreases, a higher power input to nebulize the liquid is required. This trend may be expected as the ID decreases, the thickness of the capillary wall increases. Based on our previous VSSI study, the vibration at the sharp-edge is critical to the nebulization phenomenon. A thicker wall will result in less efficient vibration at the tip so that higher

power input is required. While 250- μm ID capillary has the thinnest wall, it is more fragile than other capillaries and less optimal when handling low flow rates (< 10 $\mu\text{L}/\text{min}$). Despite the slight difference in power requirement, we selected the 100- μm ID capillary as the optimal capillary for our experiments.

Another important characteristic of cVSSI is the permissible flow rates. With 100- μm ID capillary, we tested flow rates ranging from 1 $\mu\text{L}/\text{min}$ to 1 mL/min. cVSSI can nebulize liquid sample at all the flow rates in this range with the appropriate power input. At 1 $\mu\text{L}/\text{min}$, the power requirement for nebulizing fluid is ~ 130 mW. As the flow rate increases to 20 $\mu\text{L}/\text{min}$, the power requirement increases to ~ 260 mW. At 1 mL/min, it requires ~ 760 mW power to form stable plume. These experiments demonstrate cVSSI can work over a wide range of flow rates to accommodate different experimental setups. Even at the only tested high flow rates (~ 1 mL/min), less than 1 W of power consumption is still reasonable. Here, the flow rate range (1 $\mu\text{L}/\text{min}$ to 1 mL/min) is mainly limited by the capillary dimension rather than cVSSI. Using 250- μm ID capillary, we achieved a ~ 2.8 mL/min flow rate using cVSSI. For flow rates < 1 $\mu\text{L}/\text{min}$, a smaller ID capillary is required as the current 100- μm ID capillary is prone to instable flow with common syringe pumps. Collectively, we demonstrate a novel method for direct fluid nebulization from the outlet of a capillary without the need of a nebulization gas or electric field.

Characterization of cVSSI-MS

After optimizing the operational parameters of cVSSI, we characterized the ionization performance of cVSSI in MS analysis. A series of solutions containing a small molecule, a

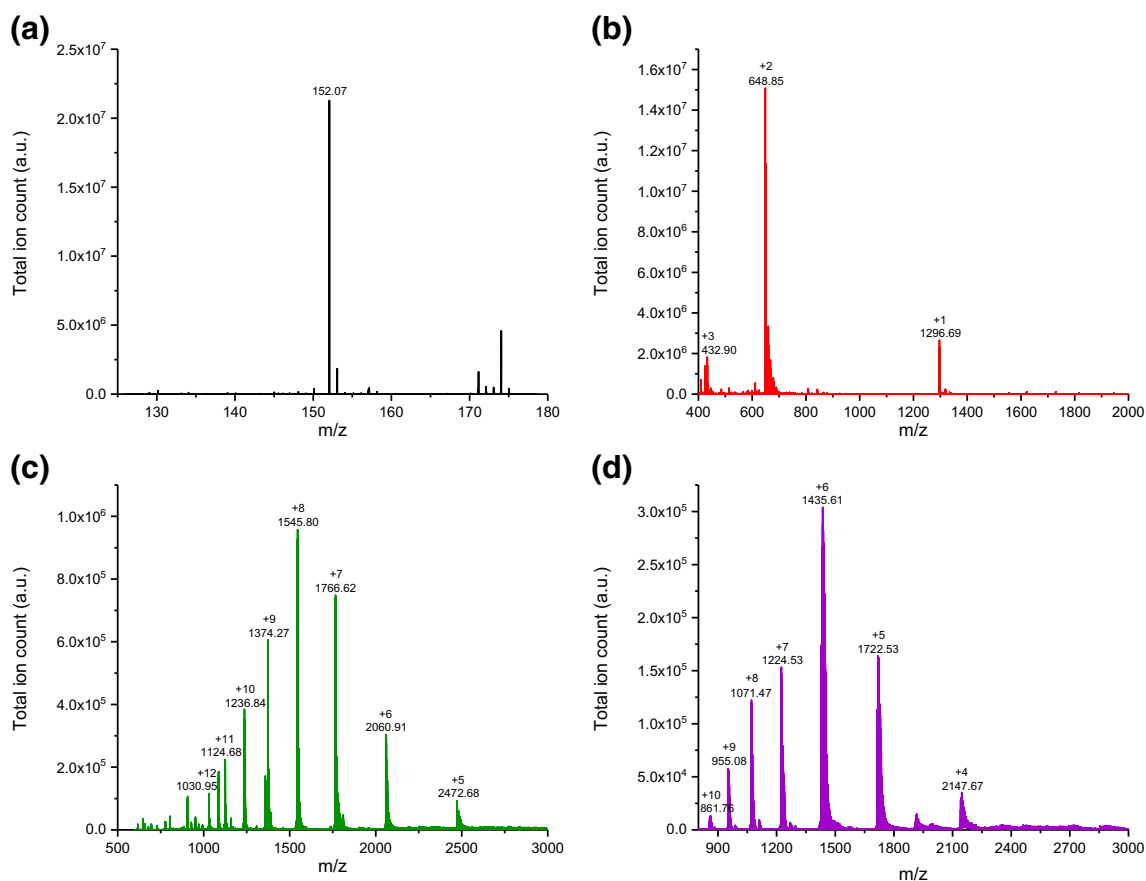


Figure 3. (a) Mass spectrum of acetaminophen solution in water:acetonitrile (1:1) at 20 $\mu\text{L}/\text{min}$. (b) Mass spectrum of 10 μM solution of angiotensin I in water. (c) Mass spectrum of 10 μM solution of cytochrome c in water. (d) Mass spectrum of 10 μM solution of ubiquitin in water

peptide, and proteins was tested using cVSSI-MS. The flow rate was set at 20 $\mu\text{L}/\text{min}$ for all of the measurements. As shown in Figure 3, 10 μM solutions of acetaminophen, angiotensin I, cytochrome c, and ubiquitin in either MeCN/ H_2O (1:1) or in pure H_2O solution resulted in successful analyte detection with cVSSI-MS. The mass spectra are consistent with those obtained using VSSI in our previous work, both of which present ESI-like spectra. For angiotensin I, a charge state distribution ranging from +1 to +3 was observed with pure water as the solvent, and +2 is the dominant peak. After adding 0.1% formic acid to the water solution, the relative abundance of the +3 peak increased significantly while the +2 feature is still the most dominant peak (Fig. S1). For protein solutions, ESI-like charge state distributions were obtained with +5 to +12 charge states for cytochrome c and +4 to +10 ions for ubiquitin. We also observed charge state reduction when adding 10 mM ammonium acetate to the ubiquitin solution (Fig. S2). The charge state distribution shifted from +4 to +10 in water to +3 to +6 in the ammonium acetate solution. Collectively, these results show that the ionization characteristics observed for VSSI are maintained in cVSSI, despite the fact that droplet nebulization occurs at the capillary outlet in cVSSI as opposed to the edge of a glass slide.

While cVSSI produces similar mass spectra as VSSI, capillary-based direct injection in cVSSI enables more stable

and convenient injection for quantitative analysis. We examined the total ion chromatogram of a 0.5-min cVSSI injection (Figure 4a). A stable injection was achieved with a CV of 4.5% observed for the ion current. The stable injection feature of cVSSI facilitates quantitative analysis of target molecules. To characterize the performance of such quantitative analyses using cVSSI-MS, different concentrations (1 nM–100 μM) of acetaminophen in MeCN/ H_2O (1:1) were prepared and tested. As shown in Figure 4b, a linear response over 5 orders of magnitude for the acetaminophen solution is obtained, demonstrating the feasibility of using cVSSI for rapid, voltage-free quantitative analysis. We further determined the limit of detection (LOD) for acetaminophen solution with the present embodiment of cVSSI using the mean intensity of the blank solution plus 3 times standard deviation as the threshold (Figure 4c). The LOD for acetaminophen was then determined to be ~ 3 nM. The sensitivity of cVSSI should be sufficient for many voltage-free MS analyses, and can be further boosted when coupling to an electrical field.

Continuous Flow Separation and Ionization Using LC-cVSSI-MS

As it is demonstrated that cVSSI can nebulize liquid sample directly at the outlet of a capillary under a wide range of flow rates, it should also be feasible to couple with HPLC for LC-

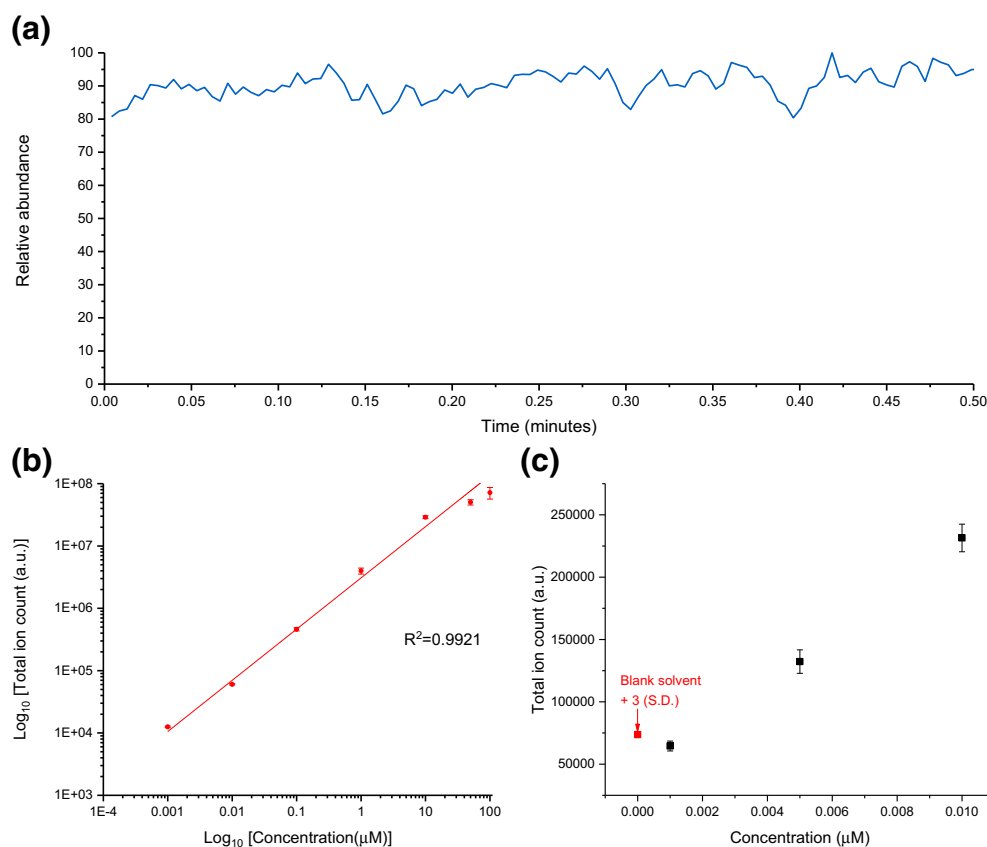


Figure 4. (a) Relative abundance of ions detected using the cVSSI source for 30 s. (b) Linear curve for acetaminophen solutions (1 nM to 100 μ M) in water:acetonitrile (1:1). (c) Blank solvent and 1 nM, 5 nM, and 10 nM acetaminophen solutions in water:acetonitrile (1:1)

MS analysis. To accommodate the high-pressure pump in HPLC and avoid leakage, we employed a capillary connector to connect cVSSI with the HPLC fluidic system (Figure 1d). The addition of the capillary connector does not affect the performance of cVSSI. With the HPLC pump, the plume can be properly generated under a 500 μ L/min flow rate. While cVSSI can handle the 500 μ L/min flow rate, we employed a 10:1 flow splitter to reduce the amount of waste generated around the inlet of the mass spectrometer for the actual LC separation experiment. We tested two common LC-MS applications with the cVSSI interface: (1) LC-MS separation and detection of compounds in a mixture of metabolites and (2) bottom-up proteomics identification of peptides using a protein digest. The mass range used for separation and detection of metabolites is between 100 and 500 m/z and for the peptide digest is between 200 and 2000 m/z .

In metabolomics, LC-MS is a powerful tool to process and analyze complex biological samples. We selected a series of representative metabolites including endogenous metabolites (L-valine, dopamine, guanosine, guanine), and exogenous metabolites (sucrose, acetaminophen, caffeine, aspartame, and benzoic acid). We prepared the mixture of these nine molecules in water each at \sim 100-ppm concentration and introduced a sample onto a reversed-phase C18 HPLC column at a flow rate of 500 μ L/min. Water with 0.1% formic acid and methanol was used as the

aqueous and organic phases, respectively. The eluent was introduced directly onto the cVSSI device through a capillary connector and detected using an Orbitrap mass spectrometer. Using protonated mono isotopic masses for the following metabolites: L-valine (118.0868), dopamine (154.0868), guanosine (284.0995), guanine (152.0572), sucrose (343.1240), acetaminophen (152.0712), caffeine (195.0882), aspartame (295.1294), and benzoic acid (123.0446), extracted ion chromatograms for the same species are plotted using Excel. Extractions to text files were performed using the XCalibur Software Suite (ThermoScientific). Notably, a number of ions have overlapping retention times; however, the high-resolution mass spectrometer allows the extraction of ion chromatograms showing distinct features. To clearly present the LC-MS results, a selected ion chromatogram is used here as the signal intensity of cVSSI is approximately tenfold lower than that of ESI. As shown in Figure 5, most of the metabolites were separated and detected by LC-cVSSI-MS. Guanosine and guanine were not separated under the present LC separation settings, but both were detected with cVSSI-MS. Even though only limited attention has been given to cVSSI design and optimization (e.g., increasing the number of droplets entering the mass spectrometer inlet), the signal levels are only about tenfold lower than those obtained from ESI (Figure S3). This is noteworthy considering that ESI is aided by heated bath gases and has experienced many years of source engineering. It is also possible that the lowered

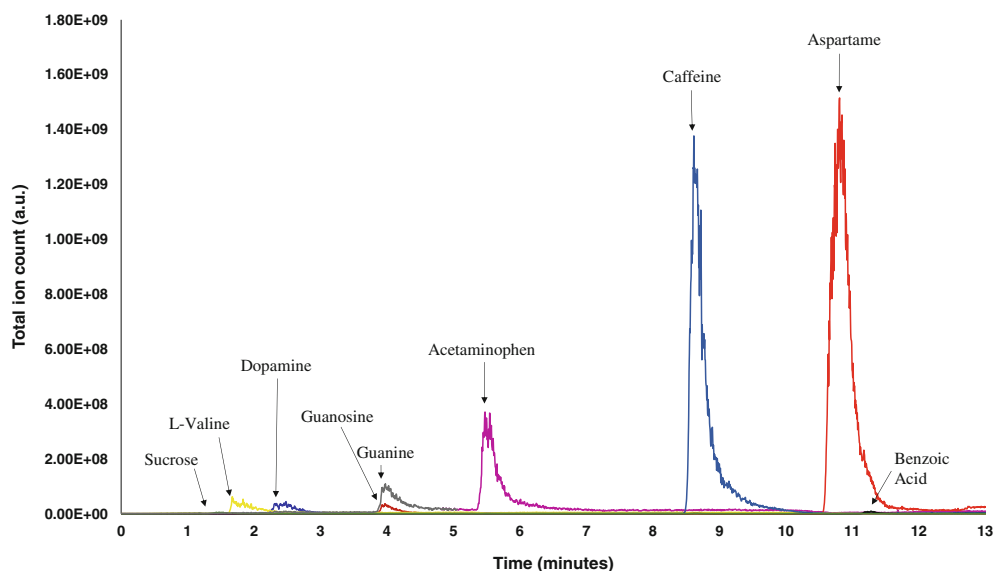


Figure 5. LC-cVSSI-MS separation of a metabolite mixture containing sucrose, L-valine, dopamine, guanosine, guanine, acetaminophen, caffeine, aspartame, and benzoic acid each at ~ 100 -ppm concentration

average charge of the droplets decreases the overall ionization. That said, the elution times for both systems agree well. In both systems, less polar molecules such as caffeine and aspartame show higher signal intensities. It appears that ionization by cVSSI is more selective for less polar compounds compared with ESI. For instance, the intensity ratio between the less polar caffeine and L-valine is ~ 20 in cVSSI, whereas the ratio is ~ 3 in ESI. The ionization efficiency of voltage-free cVSSI tends to be affected more by the inherent polarity of the target molecules. A slight peak broadening was observed in cVSSI compared with the ESI method. The full width at half maximum (FWHM) for caffeine is 0.19 and 0.15 min in cVSSI and ESI, respectively. For aspartame, FWHM is 0.31 and 0.20 min in cVSSI and ESI, respectively. The slight peak broadening in cVSSI could be caused by the less optimal fluidic connections and the extended tubing length, which can be further improved with a dedicated fluidic system design. The tailing effect observed in cVSSI is consistent with the tailing observed in the ESI chromatogram (Figure S3). Notably, features having smaller retention time exhibit increased broadness and tailing. Because peak tailing can result from column degradation [35], it is possible that more polar sites on the stationary phase have a greater effect on retention of the more polar compounds. Additionally, some of the peak broadness may be attributed to the LC separation method. Overall, cVSSI can be coupled to HPLC systems exhibiting a wide range of flow rates and solvent systems and this work demonstrates its utility in LC-MS analysis of metabolite mixtures.

Another common application of LC-MS is peptide identification of molecules obtained from a protein digest. In the following study, LC-MS/MS experiments were conducted for a peptic digest of cytochrome *c* to test the suitability of cVSSI-MS/MS for peptide identification.

After LC-cVSSI-MS/MS analysis, peptides were identified by a protein database search. Overall, $\sim 75\%$ sequence coverage was obtained for the cytochrome *c* protein using the protein

database approach. The incomplete sequence coverage could result from low-quality MS/MS spectra for some peptides or from the fact that the precursor ions for some peptides containing the missing sequences simply are not selected for MS/MS analysis. To determine whether or not peptide assignments are missed by the protein database search, the ion chromatogram was searched manually. Here, the ion signal was averaged over retention time (t_R) windows of 2 min in duration.

From the step-wise precursor ion analysis, a total of 78 peptides are identified. These include species ranging in size from 2 to 29 amino acid residues and in charge state of +1 to +6. Several heme-containing peptides are also observed. Considering the sequences of these peptides, 100% sequence coverage of the protein is demonstrated by the peptides shown in Table S1. In general, cVSSI is shown to be sufficiently efficient for the ionization of peptides in proteomics studies. Here, it is noted that a split-flow geometry was employed. Notably, cVSSI can be conducted at much higher flow rates; however, because there is no impetus for the large number of droplets to enter the MS inlet beyond gas-flow entrainment, a tenfold split was employed. This helped to maintain the cleanliness of the source. Indeed, it may be argued that significantly higher sensitivity could be realized by cVSSI with an ion source design optimized for cVSSI's highly efficient droplet production at high flow rates. That said, at this early stage of development, cVSSI is demonstrating similar performance capabilities to ESI with 100% peptide coverage which has an advantage of being able to utilize a drying, heated bath gas.

Conclusions

We demonstrate the utility of cVSSI in continuous flow-based MS analysis and in combination with LC-MS. The simplicity, flexibility, small footprint, and low power consumption of

cVSSI make it an attractive ionization strategy for MS analysis and for in-droplet chemistry. We expect it will be especially useful for analyzing sensitive and fragile molecules that are sensitive to electrochemical oxidation or study biomolecules in their native states. Future studies will include elucidating-specific nebulization and ionization mechanisms for cVSSI and VSSI, further reducing the droplet size generated by cVSSI, and optimizing the fluid connection for LC-cVSSI-MS system.

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